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Epigenetics and the control of multicellularity

Workshop on Chromatin at the Nexus of Cell Division and Differentiation

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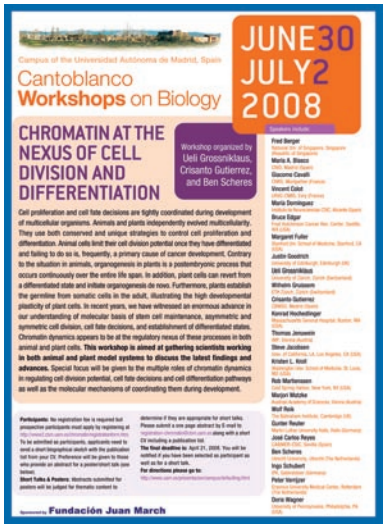

The Cantoblanco Workshop on Chromatin at the Nexus of Cell Division and Differentiation took place between 30 June and 2 July 2008, in Cantoblanco, Madrid, Spain, and was organized by U. Grossniklaus, C. Gutierrez and B. Scheres.

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See Glossary for abbreviations used in this article.

Introduction

At many points during development and adult life, cells have to react to regulatory cues, and decide between self-renewal and entering terminal differentiation. To do so, they integrate many inputs from their environment and their own developmental programme. This workshop aimed to explore the role of chromatin and epigenetics in the coordinated regulation of cell division and differentiation. The conference gathered researchers from various fields of epigenetics who work with different model systems, including yeast, *Drosophila*, vertebrates and *Arabidopsis thaliana*. They presented mostly unpublished results on a wide range of epigenetic mechanisms that are involved in the control of proliferation and differentiation, highlighting many fundamental roles of the chromatin components in these processes; this made for a truly exciting scientific programme.

Self-renewal versus differentiation

Several adult stem-cell systems have been identified in *Drosophila*, providing exceptionally favourable models for the analysis of the dynamic self-renewal of short-lived terminally differentiated cell types. B. Edgar (Seattle, WA, USA) showed that transiently ablated enterocysts induce intestinal stem-cell (ISC) proliferation after the expression of the caspase activator Reaper or the JNK activator Hemipterous, resulting in rapid intestinal regeneration. The data support a homeostatic feedback control between enterocysts and ISCs through the JAK/STAT and JNK signalling pathways. By using a *Pseudomonas entomophila* infection model, Edgar found that, besides NOTCH, JAK/STAT and JNK, the EGFR signalling pathway also controls ISC proliferation; this indicated that the ISC niche is also partly provided by progeny enterocysts ensuring the homeostatic control of ISC proliferation in response to stress, injury or infections.

M. Fuller (Stanford, CA, USA) discussed another powerful system with which to study the control of stem-cell self-renewal and differentiation: *Drosophila* male adult germ-line stem cells (GSCs). Signalling from the adjacent somatic niche cells activates the STAT transcription factor in GSCs and in adjacent somatic stem cells to control self-renewal. Differentiation from mitotically proliferating precursor cells to spermatocytes is marked by major transcriptional changes. Testis-specific TATA box binding protein-associated factor (tTAF) and testis-specific meiotic arrest complex (tMAC) components, which are required for spermatid differentiation, antagonize silencing mediated by Polycomb proteins (Schuettengruber *et al*, 2007). Fuller and co-workers had previously found that tTAFs counteract silencing mediated by Polycomb components of the PRC1 to activate testis-specific genes (Chen *et al*, 2005). They have now shown that the E(z) and Su(z)12 components of the PRC2 are highly expressed in precursor cells but become abruptly downregulated during differentiation, whereas Pol II is recruited to differentiation genes and tTAFs are turned on in early spermatocytes. The data indicate a multistep process involving the

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Glossary

AP2	apetala 2
bHLH	basic helix–loop–helix
CDT1	ARABIDOPSIS HOMOLOGUE OF YEAST CDT1 (that is, cell-division cycle 10-dependent transcript 1)
CDX2	caudal type homeobox transcription factor 2
CENH3	centromeric histone H3
CHG	indicates a sequence of DNA, in which H may be A, C or T
ChIP	chromatin immunoprecipitation
CMT3	chromomethylase 3
DDM1	decrease in DNA methylation 1
DNMT1	DNA (cytosine-5)-methyltransferase 1
DRM	DRM DOMAINS REARRANGED METHYLASE
EGFR	epidermal growth factor receptor
Elf5	E74-like factor 5
ESET	ERG-associated protein with SET domain
GEM	GLABRA2 expression modulator
GL2	*GLABRA2*
HMT	histone methyltransferase
HP1	heterochromatin protein 1
JAK	janus kinase
JNK	c-Jun amino-terminal protein kinase
KLF4	Kruppel-like factor 4
kyp	KRYPTONITE (SUVH2)
miR	microRNA
OCT4	Octamer-binding transcription factor 4
PcG	Polycomb group
PLT	plethora
Pol II	RNA polymerase II
PRC	polycomb repressive complex
RPB	retinoblastoma related
SDC	Suppressor of DOMAINS REARRANGED METHYLASE-dependent cytosine methylation
siRNA	small-interfering RNA
SOX2	Sry-related HMG box
STAT	signal transducer and activators of transcription
Suv4-20h	Suppressor of variegation 4-20 homologue
SUVH	SU(VAR)3–9 homologue 1
SWI/SNF	switch/sucrose nonfermentable
TGS	transcriptional gene silencing
TRF1	telomeric repeat-binding factor
TTG1	TRANSPARENT TESTA GLABRA 1
UAS	upstream activating sequence

removal of Polycomb complexes from chromatin, and the binding of tTAF and tMAC components to activate transcription.

In plants, the root stem cells reside in niches and are maintained by short-range signals. In *Arabidopsis*, the PLT AP2-domain transcription factor is required for root stem-cell specification. B. Scheres (Utrecht, The Netherlands) discussed the involvement of polar auxin transport in this phenomenon (Galinha *et al*, 2007; Grieneisen *et al*, 2007), and showed that the auxin hormone induces PLT expression and that the PLT protein dosage is translated into distinct cellular responses. The RPB protein is involved in this process through a complex molecular connection with upstream stem-cell specification factors.

Additional chromatin components that regulate the choice between self-renewal and differentiation are the members of ATP-dependent chromatin-remodelling complexes. Neuronal cell fate and differentiation is controlled by the Neurogenin and NeuroD bHLH proteins, which interact directly with the SWI/SNF ATP-dependent chromatin-remodelling complex. Geminin is a new nuclear factor that blocks neuronal differentiation by antagonizing bHLH factor and SWI/SNF function (Seo *et al*, 2005). K. Kroll and co-workers (St Louis, MO, USA) defined genome-wide bHLH target genes and resolved a direct role of Geminin in the control of bHLH protein binding to their enhancer consensus sequence (Seo *et al*, 2007). Overexpression of Geminin blocks bHLH binding to enhancers, which correlates with a reduction in histone H3K9 acetylation at these sequences. Therefore, the competition between bHLH and SWI/SNF components versus Geminin might drive the balance between stem-cell proliferation and neurogenesis.

In plants, the coordinated balance between cells that are undergoing either the cell cycle or the differentiation-associated endocycle is of special importance. C. Gutierrez (Madrid, Spain) presented results on the functional analysis of components of the pre-replication complexes that control the decisions between cell proliferation, cell fate and the differentiation-associated endocycle. Root development is regulated by GEM, which interacts with the pre-replication complex component CDT1 and with the transcriptional regulator of cell-fate genes TTG1. GEM controls the level of histone H3K9 methylation on the promoters of the *GL2* gene (Caro *et al*, 2007). These results are reminiscent of the dual function of Geminin in animal cells (Caro & Gutierrez, 2007), and point to a crucial role of chromatin remodelling in the control of cell division and cell fate.

Epigenetic processes have a crucial role in plant seed development. The identification of maternal effect genes illustrates the importance of parent-of-origin effects; moreover, studies in *Arabidopsis* and maize have shown that, for many genes, no transcripts derived from the paternal allele can be detected during the first days after fertilization (Grimanelli *et al*, 2005; Vielle-Calzada *et al*, 2000), suggesting preferential maternal control over early seed development. However, the paternal alleles of some genes are expressed comparatively early, indicating that the time of paternal activation might vary between different loci (Meyer & Scholten, 2007; Weijers *et al*, 2001). U. Grossniklaus (Zürich, Switzerland) reported on the epigenetic control of paternal genome activation during embryogenesis in *Arabidopsis*. Analysis of reporter lines and endogenous genes revealed a delayed activation of paternal alleles in both the embryo and the endosperm. Paternal activation occurs gradually, with each of the loci studied showing distinct timing and kinetics of activation, and is dependent on maternal factors, which define epigenetic pathways that both repress and activate paternal alleles.

A common denominator in these reports was the identification of the complexity in epigenetic gene regulation. Epigenetic components—once regarded as constitutive silencers or activators of gene expression—are beginning to reveal their ability to build modular regulatory systems that can be switched, but also tuned, in response to a wide range of developmental and environmental cues.

Epigenetic landscapes defining chromatin states

The genome-wide analysis of epigenetic components and chromosomal landscapes was discussed in several talks. In mammalian cells, H3K9me3 is generally regarded as a mark of heterochromatic regions that are located in the pericentromeric parts of the chromosomes and depend on the activity of the Suv39h HMTs. However, a tiling array analysis across mouse chromosome 17, described by T. Jenuwein (Vienna, Austria), revealed two subpopulations of H3K9me3 enrichments along the euchromatic chromosome arms. One of these reflects heterochromatic islands, where the H3K9me3

mark depends on the Suv39h enzymes and correlates with the presence of double-stranded RNAs (dsRNAs) and components of the RNA interference (RNAi) machinery. The second class of H3K9me3 enrichments is independent of Suv39h function, does not correlate with dsRNA, and is marked by the presence of the ESET HMTase and by HP1α. A comparison of proliferating embryonic stem (ES) cells, ES-derived erythroblasts and mouse embryonic fibroblasts (MEFs) revealed a correlation between the gene-density map and Suv39h-independent H3K9me3 enrichments, suggesting a function in transcriptional read out. By contrast, the genomic position of Suv39h-dependent heterochromatic islands cannot be predicted by a gene-density map and is highly variable in the chromatin of different cell types. It will be interesting to determine the function of this plastic portion of the epigenome and to explore whether it can be used as a fingerprint of cellular states.

Heterochromatin components are involved not only in the control of centromeric heterochromatin and heterochromatin islands, but also in the regulation of telomeres. M. Blasco (Madrid, Spain) discussed the epigenetic regulation of mammalian telomere physiology. At mammalian telomeres, Dicer-dependent miR-290 is involved in the control of telomere length (Benetti *et al*, 2008). Telomere transcripts of 1–6 Kb in length are detected in adult mouse tissue, are conserved and can also be detected in zebrafish. They are polyadenylated, Pol II-dependent and colocalize with the TRF1 telomere-binding protein (Schoeftner & Blasco, 2008). In Suv4-20h-null MEF cells, transcription at telomeres is enhanced, implying a direct influence of the epigenetic status of telomeres on their transcription. Furthermore, telomere length correlates with the amount of telomeric RNA, and *in vitro* studies have shown that telomeric RNA might control telomerase activity. Interesting new data were also presented on telomere length and the efficiency of induced-pluripotent stem (iPS)-cell production, and on epigenetic reprogramming of telomeres in iPS cells. With a newly developed technique quantifying telomere length in individual cells, the investigators were able to show that the longest telomeres are found in stem-cell niches (Flores *et al*, 2008).

Epigenetic reprogramming of cell fates is a topic of great interest, both for the understanding of development and for therapeutic applications. In mammals, ectopic expression of the transcription factors OCT4, SOX2, c-Myc and KLF4 generates iPS cells from fibroblasts. Characterizing the epigenetic state of iPS cells, K. Hochedlinger and co-workers (Boston, MA, USA) showed that female iPS cells undergo reactivation of a somatically silenced X chromosome (Maherali *et al*, 2007). Genome-wide analysis of H3K4 and H3K27 trimethylation revealed a comparable pattern between iPS and ES cells. By using doxycycline-inducible lentiviruses, it could be shown that expression of the four genes is required for more than 8 days until the cells become self-sustaining iPS cells in the mouse, whereas 28–30 days are required in human iPS cells (Maherali *et al*, 2008; Stadtfeld *et al*, 2008). Epigenetic reprogramming during normal mammalian development occurs in preimplantation embryos, and includes active and passive DNA demethylation. W. Reik (Cambridge, UK) discussed the control of lineage commitment between ES cells and trophectoderm stem (TS) cells through differential DNA methylation. Essential for trophoblast differentiation is the activity of ELF5 (Donnison *et al*, 2005), which is highly methylated in ES cells. The *ELF5* gene is not methylated in the DNMT1$^{-/-}$ ES cells and consecutively becomes activated, resulting in the activation of the CDX2 and EOMES trophoblast-determination genes in ES cells. These studies resolved a basic pathway controlling epigenetic regulation that is crucial for commitment to the inner cell mass and the trophectoderm lineage (Farthing *et al*, 2008; Ng *et al*, 2008).

Epigenetic-landscape mapping in *Arabidopsis* was discussed extensively, providing evidence for regulation of cellular memory, as well as developmental plasticity. S. Jacobsen (Los Angeles, CA, USA) discussed the role of chromatin determinants of epigenetic landscapes in plants. In *Arabidopsis*, 10 different SU(VAR)3–9 homologues have been identified, implying functional redundancy of histone H3K9 methyltransferases. Beside the SET domain, which catalyses histone methylation, these proteins contain an SRA (YDG) domain that is implicated in binding to methylated DNA. Recent studies have indicated a positive-feedback loop between histone H3K9 methylation and CHG DNA methylation that is catalysed by the chromomethylase CMT3. The H3K9 methyltransferase KRYPTONITE (SUVH4) binds to CHG-methylated DNA, and CMT3 binds to methylated H3 histone tails. Asymmetric CHH DNA methylation is maintained by the targeting of the DRM2 DNA methyltransferase and small RNAs to the DNA. Furthermore, it was shown that the SUVH2 histone methyltransferase targets the DRM1 and DRM2 DNA methyltransferases. Triple-mutant *kyp suvh2 suvh9* plants—similar to *cmt3 drm1 drm2* mutants—have a small plant phenotype, which seems to be caused by the reactivation of the *SDC* gene. Indeed, overexpression of SDC causes a similar phenotype.

The association of the propagation of a DNA-methylation pattern through cell division is the best-known example of epigenetic memory. V. Colot (Paris, France) reported the inheritance of a large sample of hypomethylated sequences resulting from DDM1 loss of function in *Arabidopsis*. The progeny of plants that no longer carry the *ddm1* mutation have been followed for up to eight generations. Remarkably, although stable hypomethylation was found at some sequences, which is consistent with previous observations (reviewed in Richards, 2006), efficient and faithful remethylation was observed at others. The analysis of this process indicated an essential role of the RNAi-dependent machinery in the distinction between the two types of sequence.

R. Martienssen (New York, NY, USA) discussed the RNAi-dependent mechanisms that control heterochromatin formation in *Schizosaccharomyces pombe* (Kloc *et al*, 2008) and *Arabidopsis*. By using a series of ura4 insertions at various sites within the centromeric heterochromatin of *S. pombe*, it was possible to show that RNAi formation is essential for the spreading of the H3K9me2 histone-methylation mark, but not for the recruitment of the H3K9 methyltransferase clr4. Furthermore, silencing depends on the HP1 homologue Swi6p. In swi6-mutant cells, silencing is impaired, although H3K9 methylation and RNAi production are not significantly reduced. After synchronizing the cells, Martienssen could show oscillation of H3K9 methylation, H3S10 phosphorylation, siRNA accumulation and Swi6 chromatin association during the cell cycle. Binding of Swi6 to chromatin was directly affected by H3S10 phosphorylation. Performing comparable studies in *Arabidopsis* cell cultures, in which most of the cells are in G1, Martienssen found significant cell-to-cell differences in DNA methylation at retrotransposons, which correlate with considerable changes in small-RNA production. In cell culture, transposons become demethylated and are expressed, whereas other sequences gain DNA methylation. These processes are clonally induced and then stably maintained, indicating that new epialleles might be frequently generated during cell culture.

Fig 1 | Epigenetic triggering of cancer progression in *Drosophila melanogaster*. (**A**) Control eye size. (**B**) *Delta*, *pipsqueak* and *lola* overexpression (the eyeful phenotype), showing eye tumour growth. (**C**) Eye-derived secondary growth (red tissue mass) and disseminated tumour cells into the gut. The image shows an open abdomen of an eyeful fly. Images kindly provided by M. Dominguez.

Kinetochore protein complexes at eukaryotic centromeres are responsible for the correct segregation of chromosomes during nuclear divisions. Kinetochore formation is epigenetically regulated and initiated by the substitution of histone H3 with CENH3 within centromeric nucleosomes. In contrast to the case in humans and *Drosophila*, CENH3 loading in *Arabidopsis* and barley occurs during (late) G2 of interphase, when two sister kinetochores become detectable. Data from the laboratory of I. Schubert (Gatersleben, Germany) showed that the histone fold domain of the carboxy-terminal part of CENH3 is sufficient to target *Arabidopsis* centromeres (Lermontova *et al*, 2006). Recent work shows that partial RNAi-mediated depletion of CENH3 causes dwarfism by growth reduction based on a reduced number of mitotic divisions rather than on reduced cell growth.

In summary, these reports illustrate the rapid progress that we are witnessing in the area of chromatin genomics, and the partial convergence between animals and plants in many regulatory aspects of chromosome biology. The challenge for future studies will be to gain the ability to perform epigenomic mapping in specific cell types. This will allow a functional analysis of chromatin dynamics during cell differentiation in living organisms.

Mutational dissection of epigenetic processes

In order to understand the role of the various components of the epigenetic machinery in setting up and modulating chromatin landscapes during development, genetic dissection of these processes is of paramount importance. By using transgene systems that monitor various molecular mechanisms of gene silencing, large-scale screens for silencing suppressor mutations have been conducted in *Arabidopsis*.

M. Matzke (Vienna, Austria) reviewed a molecular analysis of new factors controlling RNA-dependent gene silencing, which could be identified with the help of a transgene containing viral enhancers that are active in the root and shoot meristem. In the transgene, spreading of silencing depends on the production of siRNAs (Kanno *et al*, 2008). The screen identified interesting new proteins, including a new structural-maintenance-of-chromosomes hinge-domain-containing protein, which functions either in stabilizing the interaction of siRNA with DNA or in the control of Pol IV function (Kanno *et al*, 2008).

G. Reuter (Halle, Germany), presented the results from another large-scale screen for chromatin components. More than 30 suppressor mutations for TGS were isolated using a transgene with tandem repeats of the luciferase reporter gene. The analysis of the corresponding genes identified several new heterochromatin-associated proteins, implying that TGS of the luciferase transgenes is caused by heterochromatinization. ChIP showed that H3K9 and DNA methylation at the transgenes are an essential, but not sufficient, prerequisite for gene silencing.

Finally, genetic alterations of epigenetic components can perturb development and give rise to diseases such as cancer. M. Dominguez (Alicante, Spain) designed an experimental system to identify molecular mechanisms of aberrant epigenetic control of tumour-suppressor genes in *Drosophila*. The investigators identified a UAS P-element insertion that, after eyeless–GAL4-mediated activation in the eye, produces metastatic tumours in the presence of hyperactive NOTCH signalling (Fig 1). Tumour formation depends on the ectopic activation of the *pipsqueak* gene, which causes silencing of the tumour-suppressor gene *Retinoblastoma-family protein* (Ferres-Marco *et al*, 2006). New results were presented showing that the interaction of Pipsqueak with ubiquitin ligase complexes controls the degradation of chromatin targets. One of these targets was found to be a histone variant, suggesting a mechanism by which proliferation and de-differentiation of cells in tumorigenesis is induced by excess Pipsqueak activity through the control of histone dynamics at tumour-suppressor loci. As Pipsqueak is also involved in the function of Polycomb complexes, and as PcG genes are involved in cancer progression in mammals, it will be of interest to study the role of PcG genes in genetically defined cancer systems.

Conclusion

We have recently seen formidable progress in our molecular understanding of chromatin landscapes and function. The biochemical and functional characterizations of a swarm of chromatin-modifying complexes have greatly increased and deepened our knowledge of the regulatory functions of epigenetic components. These studies have been complemented by extensive genome-wide mapping of the same components, of modified histones and histone variants, and of methylated DNA and noncoding RNAs. We now know that several epigenetic components are involved in the maintenance of cell proliferation although, in response to differentiation signals, epigenetic regulatory programmes can change and accompany cell-fate acquisition and cessation of cell growth and proliferation. However, for technical reasons, most of the studies have been carried out in transformed cultured cells, undifferentiated stem cells or lower eukaryotes. The challenges ahead will be to extend this work to real living organisms in a true developmental context, and to analyse how epigenetic programmes can be switched in response to different stimuli, how stable they are, and how stability and plasticity of epigenetic regulation can be acquired and tuned. A remarkable feature of many regulatory pathways is that their output depends on the so-called 'context'—that is, they can induce markedly different effects in various tissues or developmental times. An exciting possibility is that epigenetic regulation during the history of different cell lineages might represent a large proportion of the set of inputs that defines this context. If this is true, epigenetics will contribute deeply to the understanding of development and disease in the coming years.

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